MATURATION OF THE REPRODUCTIVE TRACT OF *NEOHELIX MAJOR* (BINNEY) (GASTROPODA: POLYGYRIDAE)

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ABSTRACT

Maturation of the reproductive system of *Neohelix major* (Binney), from post-hatching through sexual maturity, was examined. Laboratory reared snails were used throughout this study so that ages of animals were known. Snails were sampled every 30 days starting at one month and continuing through 14 months of age. The ovotestis, hermaphroditic duct, albumen gland, spermoviduct and terminal genitalia (free oviduct, vas deferens, vagina, penis and bursa copulatrix) were examined histologically.

Considerable intraspecific variation exists in the structural development of the reproductive tract of *Neohelix major* even among animals of the same age. Within the same adult age class, animals with shells in good condition showed uniformity in development but individuals with damaged shells were variable and always immature. There is a relationship between shell diameter and degree of maturity reached by the reproductive tract. Snails four months old contained spermatogonia and spermatocytes, but mature spermatozoa did not appear until the snails were nine months old. Oocytes were present from the first month although the largest appeared at 12 months of age. Until eight months of age the reproductive tract appeared immature and male organs were more evident. At nine months old, all organs, especially those of the female, increased in size and became more voluminous. The male system completed maturation slightly earlier than did the female system. Maturity is reached for most of the animals between 10 to 12 months of age when the ovotestis already contains spermatozoa.

The reproductive anatomy of several Triodopsinae has been studied by Simpson (1901), Pilsbry (1940) and Emberton (1988). Although the biology of *Neohelix major* (Binney) has been studied by Vail (1978) during two consecutive reproductive seasons, details of the changes in the reproductive system from hatching to sexual maturation are not yet available for this species. In contrast, detailed descriptions are available for *Arion ater rufus* Linné based on material reared in the laboratory (Lusis, 1961) and collected from the field (Smith, 1966), and also for *Agriolimax reticulatus* (Muller) (Runham and Laryea, 1968; Runham, 1978).

Other authors have described various aspects of maturation of the reproductive tract and suggested the possibility of controlling factors. Laviolette (1954) described the role of the gonad in the maturation of the reproductive tract in the Arionidae suggesting hormonal control. Smith (1966) found a close relationship between maturation and seasons, and later (1967) investigated the relationship between neurosecretory activity and maturation.

This paper describes different stages in the maturation of the reproductive tract of the polygyrid gastropod *Neohelix major*, from recently hatched snails to maturity.

MATERIALS AND METHODS

This study was based upon snails reared in the laboratory by Dr. Virginia Vail. The parental stock comprising 21 mature specimens came from Greenwood Plantation, near Thomasville, Thomas County, Georgia, U.S.A. Snails were maintained under laboratory conditions over two consecutive reproductive seasons. Vail made observations on the eggs and young (Vail, 1978), and fixed snails of known ages during the summer season (June-July) so it was possible to compare not only animals of different ages but also individuals of the same age. Following a baseline study of these preserved snails, animals of known ages were sampled every 30 days, starting at one month and continuing until 14 months of age.

Generally, each sample consisted of five to seven snails but some critical ages necessitated analysis of more snails. Individuals of each age-class belonged to the same clutch of eggs. Before dissecting the animals, shell diameter and shell height were measured.

Snails of each sample were dissected and ovotestis, hermaphroditic duct, albumen gland, spermoviduct, free oviduct, vas deferens, penis, vagina and spermatheca were embedded in paraffin blocks, sectioned at 6 and 10 μm, and stained with hematoxylin-eosin or Feulgen-fast green.

In the case of younger snails, when it was not possible to dissect out certain organs, the whole animal or portions of it were embedded and sectioned. Entire structural tracts of different ages were drawn at the same magnification with the help of a camera lucida to observe qualitative differences with respect to the size of the organs. Quantification of the differences have not been practical because of the irregular shape of the organs.

RESULTS

GROSS ANATOMY

In sexually mature individuals of *Neohelix major* (Fig. 1), the ovotestis is embedded in the digestive gland. Small collecting ducts join in sequence to form the hermaphroditic duct which, at first, is narrow and straight proximally, then becomes wide and strongly coiled, finally again narrowing distally. This duct ends in the talon at the base of the large, curved albumen gland. The duct of the albumen gland joins the spermoviduct which is a long and convoluted organ that consists of incompletely separated female and male parts. At its distal portion the spermoviduct splits into two separate ducts: the vas deferens and the free oviduct. The vas deferens is a long, coiled narrow duct that continues to the proximal part of the penis, whose walls are thick and muscular. The free oviduct is short and ends by joining the spermathecal duct to form the vagina. The vagina and penis have a common gonopore located on the right side of the head.

OVOTESTIS

When fully developed the ovotestis consists of many lobes, each consisting of numerous tubules. Their number is variable and related to the size and age of the specimen.

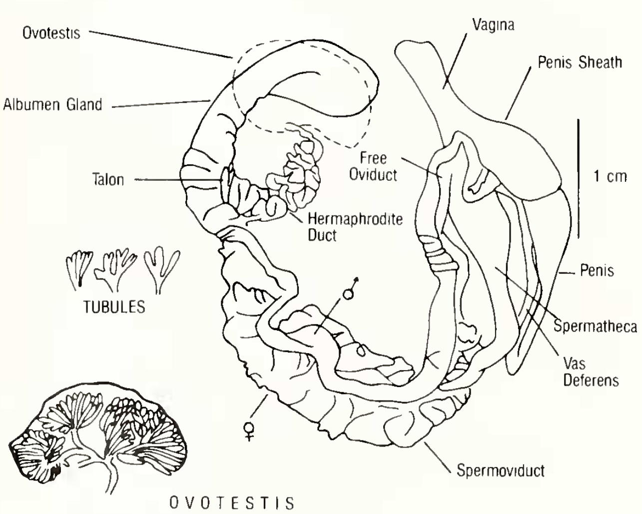


Fig. 1. Gross anatomy of the reproductive tract of *Neohelix major.*

The tubules are connected via small collecting ducts to the main hermaphroditic duct. At first, these tubules are short, but later increase in size to form longer sacs. Usually the tubules are simple but sometimes they bifurcate or trifurcate. Both male and female gametes develop in the same tubule.

Four general stages are recognized in the ovotestis: a) undifferentiated; b) spermatocyte; c) spermatid; d) spermatozoa. The undifferentiated stage extends from the first through the fourth month of life. According to Luchtel (1972), at first two types of cells form the gonad: germinal and non-germinal cells. The germinal line differentiates into either spermatogonia or oogonia and the non-germinal cells give rise to follicle and sertoli cells, the so-called "auxiliary cells" (Fig. 2). Morphological differences among germinal and non-germinal cells are difficult to distinguish with light microscopy, but it appears that non-germinal cells are peripherally positioned and germinal cells are centrally positioned in the young gonad. Also, peripheral cells are smaller and stain more deeply with hematoxylin than central ones.

At one month of age tubules are very small and almost round. By two to three months of age more tubules are visible embedded in different portions of the digestive gland; they have increased in size, mainly in length, and are completely filled with germinal cells. The spermatogonia have a small amount of cytoplasm in comparison to the size of the nucleus. Their nuclei are usually granular in appearance.

SPERMATOCYTE STAGE

The spermatocyte stage seems to extend over a considerable period of time. When snails are four months old, the tubules are densely packed, and the first spermatocytes and oocytes can be distinguished (Fig. 3). Spermatocytes have more cytoplasm than spermatogonia and the nucleus is bigger and less granular. Spermatocyte nuclei are spherical or oval in shape. Different stages of meiosis are seen among them. Usually they are placed in the lumen of the tubule while oocytes are always related to auxiliary cells in the tubule epithelium. Oocytes have nuclei with peripherally located nucleoli. They occur in groups of two to four, although they can be found alone. The tubules increase in number and size through five to seven months of age. During these months spermatocytes have also increased in size and number. Groups of them are interconnected by cytoplasmic bridges and attached to Sertoli cells located in the tubular epithelium. Spermatocytes are especially abundant when the snails are five to ten months old. At the age of eight months some oocytes show indications of vitellogenesis.

SPERMATID STAGE

The spermatid stage is reached by nine months, when the number of spermatocytes have increased considerably. Secondary spermatocytes are identifiable. Large numbers of dividing cells showing various meiotic figures are characteristic of this stage. Several stages of spermatid differentiation were observed. In early stages much cytoplasm surrounds the nucleus, which is round and compact (Fig. 4). At this point

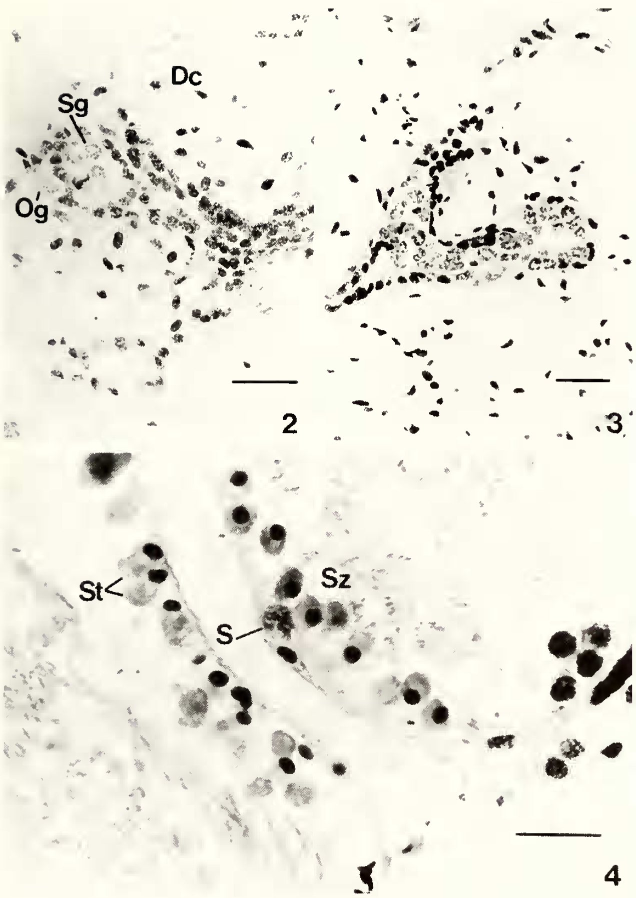


Fig. 2. Undifferentiated stage: tubule (at two months of age) showing germinal cells centrally positioned, one of them in metaphase, and non-germinal cells peripherally positioned (Dc, digestive cells; Og, oogonia; Sg, spermatogonia) (scale bar = 40 μm). **Fig. 3.** Spermatocyte stage: bifurcate tubule (at four months of age) embedded in the digestive gland. The first spermatocytes and oocytes can be distinguished (scale bar = 40 μm). **Fig. 4.** Spermatid stage: early spermatids can be distinguished in the ovotestis of snails at nine months or older. The nucleus has migrated to the periphery of the cell (St, spermatids; S, Sertoli cell; Sz, spermatozoa) (scale bar = 30 μm).

it can be difficult to differentiate secondary spermatocytes from early spermatids. Later, the nucleus migrates toward the periphery of the cell and the mass of cytoplasm is found behind the head of the spermatid. Then a depression is formed at the base of the nucleus and in some cases it is possible to see a flagellum beginning to form. In later stages the head and the cell itself become elongated. As the tail grows in length the mass of cytoplasm becomes thinner until only a remnant of cytoplasm remains in the last portion of the tail (Fig. 5).

The first spermatozoa appear by nine months, although they are not very abundant. No spermatozoa, however, were present in the hermaphroditic duct at this time. It is possible that a certain quantity of spermatozoa need to be formed before they are transferred to the acinar ductules.

SPERMATOZOA STAGE

At the age of ten months, sperm start to be released into the hermaphroditic duct. The cytoplasm of the mature spermatozoa is completely sloughed off, and the head is long and curved. The head stains with uniform intensity. The mature sperm are attached mainly in clumps to large Sertoli cells. At this time the ovotestis is large with thin walls. At 13 months of age, the spermatogenic layer is thinner and some tubules appear empty, while others still have a large number of spermatozoa. Spermatocytes are few and spermatids are present in different stages of differentiation. By 14 months of age, the diameter of the tubules has decreased, as much sperm has passed out to the hermaphroditic duct. The oocytes are large and stain lightly.

HERMAPHRODITIC DUCT

The hermaphroditic duct is a long, strongly coiled duct when fully developed. It is narrow at the proximal part when it leaves the gonad and then becomes wide but narrows again at the entrance of the proximal part of the albumen gland. Great changes in the diameter of the duct occur at different ages. Before five months of age it is a straight and narrow duct, and it shows the first signs of coiling at the spermatocyte stage, at five to seven months. During the spermatozoan stage (10-12 months) it reaches its full development and becomes thick and highly coiled.

The hermaphroditic duct is made up of a layer of ciliated, columnar epithelial cells surrounded by a thin muscular layer. A third layer of connective tissue covers the muscular one.

At ten months of age sperm formed in the ovotestis begin to be liberated into the hermaphroditic duct, which increases in diameter and appears to have thinner walls. All animals examined that were ten months or older and whose gonads were at the spermatozoa stage, had sperm in the hermaphroditic duct.

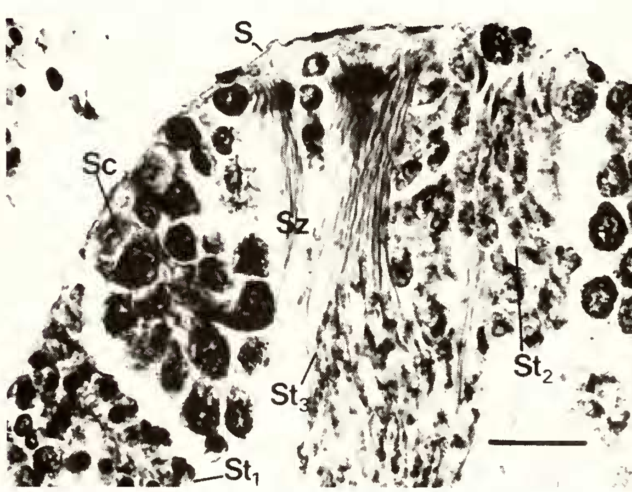


Fig. 5. Several stages of spermatid (St) differentiation in section tubule (at ten months of age). A group of spermatocytes (Sc) is attached to a Sertoli cell (S) (scale bar = 40 μm).

ALBUMEN GLAND

The albumen gland is a cylindrical organ, curved in adults, which provides the perivitelline fluids for the eggs (Rudolph, 1980). It is organized into secretory follicles that are circular in cross section. Each follicle possesses a small central duct surrounded by a single layer of columnar cells. At the base of the albumen gland exists a structure called "talon or accessory gland" by Simpson (1901), which is the "fertilization pouch" that Lind (1973) described for *Helix pomatia*. This appendage is a digitiform sac that connects with the hermaphroditic duct.

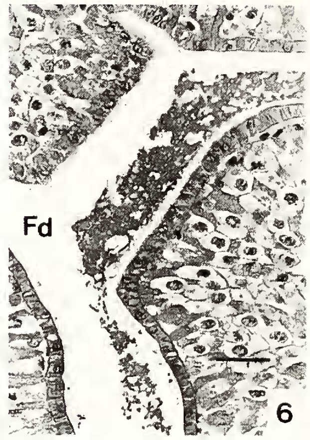
Prior to age five months, the albumen gland is a small organ consisting of a few follicles, then during the 7th and 8th months it begins to increase in size and more follicles are present.

The duct of the talon joins with the albumen gland's duct at the place where the spermoviduct starts. There appears to be direct communication between the spermduct and the groove of the talon so the sperm would not enter the oviduct groove. By 12 months of age, the albumen gland is voluminous. The secretory cells have a prominent cytoplasm in which the secretion is stored.

SPERMOVIDUCT

The spermoviduct is a glandular structure whose thickness varies with age and developmental stage of the animal. At its distal portion the spermoviduct splits into a short, free oviduct and a longer coiled vas deferens. This separation into two free ducts occurs during or shortly after the first months of the animal's life and is completed by four months of age. The spermoviduct is made up of two major components: the female and male portions. These portions are parallel to each other and are incompletely separated by longitudinal folds. The female component is composed of a wide duct lined by a thin cuboidal epithelium bearing short cilia. There are two types of glandular tissues associated with the female duct. The main one is formed by large gland cells opening individually into the lumen of the female duct via short ducts between the epithelial cells (Fig. 6). This tissue forms the characteristic folds of the spermoviduct, which are pronounced in the middle part of the organ. When glandular cells begin to produce secretion that fills their cytoplasm, they undergo a large increase in volume and the organ becomes quite thick. It is possible then to see many secretary granules liberated in the lumen of the female duct. The other female glandular tissue is composed of smaller cells and it is located at the junction of the albumen gland and the spermoviduct (Fig. 7). In 10 month old animals the cells are filled with eosinophilic secretions. According to Runham (1978) these glands could either add water to the albumen gland secretion or form the thin layer that surrounds the perivitelline fluid.

The male component of the spermoviduct is formed by a thin duct and there are three types of glands associated with it (Fig. 8). The sperm duct is much narrower and less folded than the female duct and is lined by a columnar epithelium bearing long cilia that form a thick stratum. Transport of sperm is probably carried out by ciliary action



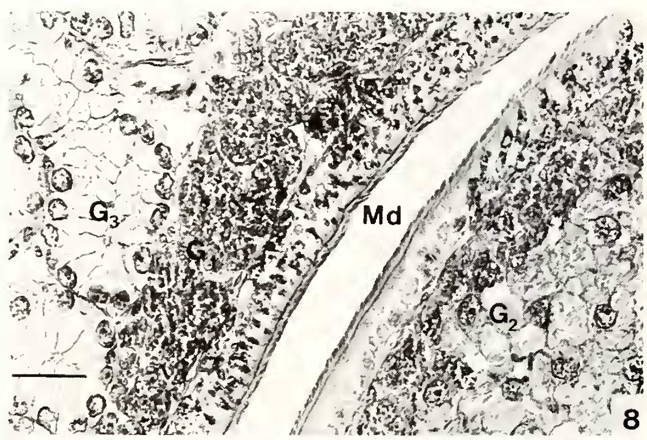


Fig. 6. Section of the female portion of the spermoviduct showing the female duct lined by a cubical epithelium bearing short cilia. This type of tissue forms the characteristic folds of the organ (Fd, female duct) (scale bar = 40 μm). **Fig. 7.** Female glandular tissue of the spermoviduct located at the junction with the albumen gland (scale bar = 40 μm). **Fig. 8.** Male part of the spermoviduct formed by a thin duct and the three types of glands associated with it (Md, male duct; G, glandular tissue) (scale bar = 40 μm).

of the epithelium. Surrounding the duct there is a gland made up of small cells which open to the lumen individually by short ducts that pass through the epithelium. Surrounding this gland there is a second type of gland. It is composed of larger cells, with individual ducts, whose cytoplasm contains large secretory granules with eosinophilic secretions. This gland is always situated between the first one and the prostate gland.

The prostate is the largest and most complex gland of the male part and is situated external to the others. It is organized into secretory follicles formed by cells radially arranged around a central lumen. The ducts of these follicles are long and discharge secretion into the lumen of the sperm duct.

The spermoviduct in *Neohelix major* does not show much differentiation until four months of age, at the beginning of the spermatocyte stage. A common duct is present, lined by a compact epithelium. The other identifiable part is the prostate gland which already has a few follicles (Fig. 9).

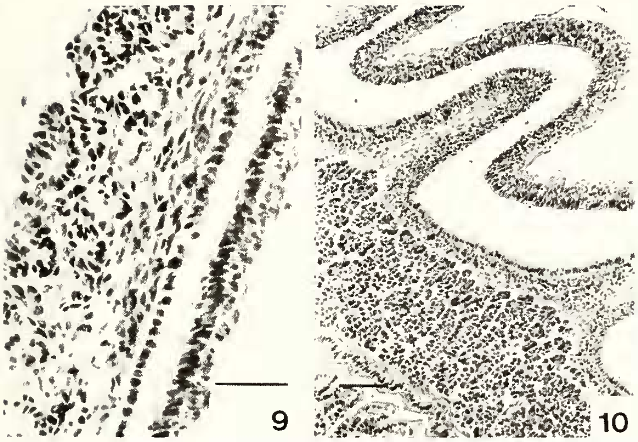


Fig. 9. Longitudinal section of spermoviduct in a five month old animal (scale bar = 40 μm). **Fig. 10.** Longitudinal section of spermoviduct in a nine month old animal (scale bar = 100 μm).

Between the epithelium and prostate gland there is a thick muscular layer which becomes thinner as the prostate grows. By eight months of age the female part has begun to fold and the two ducts are evident. The epithelium of the female duct is thick, while the male duct is composed of simple, cuboidal epithelium. The female glands are beginning to differentiate. By nine months the female portion is more folded, and the epithelium becomes thinner (Fig. 10). The cell-glands are still small. In the male side, the prostate gland is bigger but no secretion is present. In some portions of the sperm duct it is possible to see the cells that will form the other two male glands, but they are not yet clearly differentiated. At ten months of age, the cells of the prostate begin to secrete eosinophilic globules. The other two glands are already differentiated. The female part is folded but only the cell glands close to the albumen gland are full of secretions.

At 12 months of age, female and male parts are well developed, the folds are deeper, and the epithelium of the female duct is composed of cuboidal cells with short cilia. All glands have increased in size, and those of the male portion have secretions in their cytoplasm. The epithelium of the male duct is columnar and has long cilia. By 14 months all female and male glands are full of secretions and are discharging their products into the ducts. The follicles of the prostate have increased in size so much that there is no space between them. All glands reach their maximum size by this time.

TERMINAL GENITALIA

Here are included the free oviduct, vas deferens, vagina, penis and bursa copulatrix. All these organs are simple in structure with an epithelium surrounded by muscular layers. When the walls begin to differentiate and fibers form, the epithelium commences to fold. All organs then enlarge until at the spermatozoa stage they all reach their definitive size.

Separation of the spermoviduct into two free ducts, the vas deferens and the oviduct, occurs before four months old.

DISCUSSION

Maturity is defined here as the moment when sperm have been discharged into the hermaphroditic duct. All individuals that had reached their definitive shell form, with a reflected outer lip and a shell diameter of at least 30 mm, were mature. This stage is reached for most animals between ten and 12 months of age, when the ovotestis is already at the spermatozoa stage.

However, some individuals did not attain maturity until at least 12 months of age, when only spermatids, but not ripe sperm, were found in the ovotestis. Although these animals were from the same clutch of eggs and were kept under the same conditions, they developed at different rates. Usually they were smaller in size and some of them had some kind of shell damage. There is a relationship between shell diameter and degree of maturity reached by the reproductive tract, although this is not the only factor that affects development. I suggest that snails put their energy into building their shell and if something happens that damages the shell, development of the reproductive tract is stopped or slowed down, and the energy is used in repairing the shell only. This could explain the observation that all animals with damaged shells had immature reproductive tracts.

In *Neohelix major* there is a relationship between ovotestis stage and stage of maturation of the remainder of the tract. The "spermatocyte stage" means the beginning of the differentiation of the tract. At the "spermatid stage" all organs have grown and started to mature, and the "spermatozoa stage" marks the maturity of the female and male portions of the tract.

Several authors such as Laviolette (1954) and Runham *et al.* (1973), have demonstrated through different experiments on castration and transplantation of gonads or reproductive tracts, that the ovotestis influences the differentiation and functioning of the remainder of the tract. However, these kinds of experiments, such as castration, are difficult to carry out in shell-bearing snails in which the gonad is embedded in the digestive gland.

According to Boer and Joosse (1975), growth and differentiation of the reproductive tract of pulmonate gastropods is under the control of two endocrine factors produced by the gonad, one for the male and one for the female part. The production of each of these factors is controlled by hormones from the central nervous system. The male system completes maturation slightly earlier than the female system, although both male and female systems appear to be functional in the mature animal.

It would be necessary to compare this information from laboratory-reared snails with wild snails of the same species to check if maturation of the reproductive system is reached within the first year of life and if there is greater separation in time of the male and female functions. It seems that animals hatched and reared in the laboratory tend to mature more quickly than wild snails, probably because they have consistent "good" conditions of life, and hibernation or aestivation periods are shorter than in the field.

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